

1           **Gut Bacteria Metabolize Natural and Synthetic Steroid Hormones via the Reductive**  
2                               **OsrABC Pathway**

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16  
17  
18           **ABSTRACT**

19           Steroid hormone metabolism by the gut microbiome has multiple implications for mammalian  
20           physiology, but the underlying mechanisms and broader significance of this activity remains  
21           largely unknown. Here, we isolate a novel human gut bacterium, *Clostridium steroidoreducens*<sup>T</sup>  
22           strain HCS.1, that reduces cortisol, progesterone, testosterone, and related steroid hormones to  
23           3 $\beta$ ,5 $\beta$ -tetrahydrosteroid products. Through transcriptomics and heterologous enzyme profiling,  
24           we identify and biochemically characterize the *C. steroidoreducens* OsrABC reductive steroid  
25           hormone pathway. OsrA is a 3-oxo- $\Delta^1$ -steroid hormone reductase that selectively targets the  $\Delta^1$ -  
26           bond present in synthetic steroid hormones, including the anti-inflammatory corticosteroids  
27           prednisolone and dexamethasone. OsrB is a promiscuous 3-oxo- $\Delta^4$ -steroid hormone reductase  
28           that converts steroid hormones to 5 $\beta$ -dihydrosteroid intermediates. OsrC is a 3-oxo-5 $\beta$ -steroid  
29           hormone oxidoreductase that reduces 5 $\beta$ -intermediates to 3 $\beta$ ,5 $\beta$ -tetrahydro products. We find  
30           that *osrA* and *osrB* homologs predict steroid hormone reductase activity in diverse gut bacteria  
31           and are enriched in Crohn's disease fecal metagenomes. These studies thus identify the basis  
32           of reductive steroid hormone metabolism in the gut and establish a link between inflammatory  
33           disease and microbial enzymes that deplete anti-inflammatory corticosteroids.

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## 41 INTRODUCTION

42 Steroid hormones encompass a broad class of biologically active molecules that play  
43 crucial roles in diverse physiological processes. Corticosteroids, like cortisol, are involved in  
44 regulating inflammation, immune response, and metabolism,<sup>1</sup> while sex steroids, including  
45 estrogens, androgens, and progestins, regulate reproductive functions and secondary sexual  
46 characteristics.<sup>2</sup> Due to their wide-ranging effects, both natural and synthetic steroid hormones  
47 are commonly used in medical therapies to manage conditions such as autoimmune diseases,  
48 hormone deficiencies, and cancers.

49 The gut microbiome mediates diverse phenotypes through the modification of host- and  
50 diet-derived metabolites, including various steroids. Research on microbiome steroid  
51 metabolism has primarily focused on bile acids, products of which are important for multiple host  
52 phenotypes.<sup>3-5</sup> However, while less studied, steroid hormones have also been identified as an  
53 important class of substrates in the gut. These molecules interact with gut microbes after  
54 entering the gastrointestinal tract via therapeutic oral or rectal administration or through  
55 excretion in bile.<sup>6</sup> Bile is likely a particularly important source of intestinal steroid hormones, as  
56 9-22% of endogenous cortisol,<sup>7,8</sup> 10-15% of testosterone,<sup>9</sup> 20-30% of corticosterone,<sup>10</sup> and up to  
57 30% of progesterone<sup>11</sup> are eliminated from the body through this route.

58 Gut microbes generate multiple products from steroid hormones, including cortisol and  
59 corticosterone derivatives that serve as fecal biomarkers for stress in animal research  
60 studies.<sup>12,13</sup> In addition to passing into feces, intestinal microbial steroid hormone products can  
61 reenter the bloodstream through enterohepatic circulation. In humans, this is evidenced by the  
62 rectal administration of cortisol leading to an increase in specific circulating cortisol derivatives  
63 in a microbiome-dependent manner.<sup>14,15</sup> Other studies provide evidence that microbial products  
64 of progesterone metabolism similarly enter systemic circulation.<sup>6,16</sup>

65 The impact of microbial steroid hormone metabolism has been linked to several aspects  
66 of mammalian biology. Microbial inactivation of orally administered steroids, including side-chain  
67 cleavage of synthetic corticosteroids, reduces the bioavailability of these drugs.<sup>17,18</sup> Microbial  
68 pathways that dehydroxylate corticosterone and convert steroid precursors to androgens  
69 generate metabolites that contribute to hypertension in animal models<sup>19,20</sup> and promote  
70 castration-resistant prostate cancer,<sup>21,22</sup> respectively. Bacterial metabolisms that alter the  
71 concentration of steroid hormones with distinct biological activities thus have diverse  
72 consequences for mammalian biology.

73 Previous studies have reported that some gut bacteria reduce the  $\Delta^4$ -bond in steroid  
74 hormones, generating  $5\beta$ -steroid derivatives.<sup>23</sup> This activity decreases the anti-inflammatory and  
75 androgenic properties of glucocorticoids and androgens, respectively, and converts progestins  
76 into a neuroactive form.<sup>24</sup> While it stands to reason that  $\Delta^4$ -steroid hormone reduction may have  
77 implications for host biology, the molecular basis and the broader significance of activities in the  
78 gut microbiome remains unknown.

79 Here, we describe the isolation and characterization of a novel steroid hormone-  
80 metabolizing gut bacterium, *Clostridium steroidoreducens* HCS.1. By employing a  
81 multidisciplinary approach, integrating genomics, transcriptomics, and metabolomics, we  
82 characterize the *osrABC* reductive steroid hormone pathway. These findings provide new  
83 insights into the diversity of steroid hormone metabolism in the gut microbiome and its potential  
84 impact on host health.

## 85 RESULTS

### 87 *Clostridium steroidoreducens* is a novel steroid hormone-enriched gut bacterium

88 To select for members of the gut microbiome with metabolic capabilities that provide a  
89 selective advantage in the presence of steroid hormones, we passaged a human fecal sample  
90 in a nutritionally limited base medium supplemented with individual corticosteroids (cortisol or  
91 corticosterone) or sex steroids (progesterone or testosterone) (**Figure 1A**). We isolated strains

92 from the final enrichment passages and cultivated them on steroid hormone-infused solid  
93 media. We identified one strain, HCS.1, that cleared insoluble cortisol or progesterone from the  
94 solid media and accumulated a white precipitate indicative of a potential reaction product at  
95 colony centers on progesterone-infused media (**Figure 1B and 1C**). Consistent with HCS.1  
96 possessing a selective advantage in the presence of steroid hormones, 16S rRNA amplicon  
97 sequencing of the final enrichment passages revealed an amplicon sequence variant matching  
98 the HCS.1 16S rRNA sequence was enriched from below the limit of detection in the fecal  
99 inoculum to 0.7-23.6% of the microbial community following cortisol, corticosterone,  
100 progesterone, or testosterone enrichment (**Figure 1D**).

101 To facilitate further strain characterization, we sequenced and assembled HCS.1 DNA  
102 into 3 circularized contigs, comprising a genome and two plasmids, that contain 3,816,008 base  
103 pairs with 28.4% G + C content, 3595 protein-coding genes, and 111 RNA genes (**Figure 1E**).  
104 Phylogenetic analyses revealed that HCS.1 was closely related to *Clostridium chrysemydis* but  
105 represents a novel species of the genus *Clostridium*, based on an average nucleotide identity  
106 (ANI) of 92.09% to the closest reference genome and accepted taxonomic assignment criteria  
107 (**Extended Data Figure 1**).<sup>25</sup> In recognition of steroid hormone reductase activities detailed  
108 below, we assigned HCS.1 the species name *Clostridium steroidoreducens*.

### 109 **C. steroidoreducens possesses broad steroid hormone reductase activity**

110 To determine whether the *C. steroidoreducens* HCS.1 steroid clearance phenotype was  
111 indicative of metabolic activity, we employed an LC-MS-based assay to track the fate of cortisol  
112 or progesterone in *C. steroidoreducens* HCS.1 culture. We observed that both steroid hormones  
113 were fully depleted from the media, coinciding with the emergence of a minor and major  
114 product. By comparing to compound reference standards, we confirmed that major and minor  
115 products corresponded to 5 $\beta$ -dihydro- and 3 $\beta$ ,5 $\beta$ -tetrahydro-steroid derivatives, respectively  
116 (**Figure 2A, Extended Data Figure 2**).

117 Tracking *C. steroidoreducens* HCS.1 cortisol metabolism over time, we observed that  
118 5 $\beta$ -dihydrocortisol transiently accumulated, peaking at 60 minutes before decreasing to less  
119 than 2% of the total corticosteroid present by 120 minutes (**Figure 2B**). By contrast, 3 $\beta$ ,5 $\beta$ -  
120 tetrahydrocortisol steadily accumulated following the introduction of cortisol, exceeding 98% of  
121 the corticosteroid present by 120 minutes (**Figure 2B**). These results suggest that, in contrast to  
122 previously characterized bacterial steroid dehydroxylation<sup>26</sup> and side chain-cleaving<sup>27</sup> activities,  
123 *C. steroidoreducens* HCS.1 exclusively reduces cortisol, converting it to 3 $\beta$ ,5 $\beta$ -tetrahydrocortisol  
124 via a 5 $\beta$ -dihydrosteroid intermediate (**Figure 2C**).

125 To address the specificity of *C. steroidoreducens* HCS.1 steroid utilization, we next  
126 tested the strain's activity on a panel of steroids with variable functional groups at multiple  
127 positions on the sterol core (**Figure 2D**). We found that *C. steroidoreducens* tolerated  
128 substitutions at C1, C11, C17 positions, exhibiting activity on distinct corticosteroids  
129 (corticosterone, cortisone, prednisolone) and sex steroids (progesterone, testosterone) (**Figure**  
130 **2E**). In contrast to these polar steroids, the hydrophobic cholesterol-derivative cholestenone  
131 was a poor substrate (**Figure 2E**). These results establish *C. steroidoreducens* as a steroid  
132 hormone-reducing gut bacterium with broad substrate specificity.

### 133 **Fe-S flavoenzyme family OsrB is a 3-oxo- $\Delta^4$ -steroid hormone reductase**

134 We next sought to identify the mechanism of steroid hormone reduction by *C.*  
135 *steroidoreducens*. As bacterial reductases are often induced in the presence of their  
136 substrate,<sup>26,28</sup> we employed a transcriptomics-based approach to identify candidate steroid  
137 hormone reductases in *C. steroidoreducens*. We performed RNA-seq analysis on *C.*  
138 *steroidoreducens* cells cultivated in the presence or absence of cortisol and identified 30 genes  
139 that were induced >2-fold when cortisol was present (**Supplementary Table 1**). Two of the most  
140 highly induced genes, which we renamed *osrA* and *osrB* (oxosteroid reductase A and B),  
141  
142

143 encoded proteins annotated as *fadH*-like 2,4-dienoyl-CoA reductases (**Figure 3A,**  
144 **Supplementary Table 1**).

145 *E. coli* 2,4-dienoyl-CoA reductase is the best characterized member of the “Fe-S  
146 flavoenzyme family” of oxidoreductases that contain a conserved N-terminal substrate-binding  
147 domain (PF00724) and a C-terminal NAD(P)H cofactor-binding domain (PF07992) (**Figure**  
148 **3B**).<sup>29</sup> Divergent members of the Fe-S flavoenzyme family are widespread in gut bacteria and  
149 possess distinct substrate specificities for host- and diet-derived metabolites.<sup>30</sup> Consistent with  
150 OsrA and OsrB representing novel Fe-S flavoenzyme subtypes with distinct substrates, we  
151 observed that these enzymes exhibited remote sequence homology to previously characterized  
152 Fe-S flavoenzymes, including *Clostridium scindens* Fe-S flavoenzymes, BaiCD and BaiH, which  
153 reduce bile acid intermediates structurally related to steroid hormones (**Figure 3C**).<sup>29,31</sup>

154 To test steroid reductase activity of OsrA and OsrB, we heterologously produced the  
155 enzymes in anaerobically cultured *E. coli* cells. We found cells expressing *osrB*, but not *osrA*,  
156 reduced cortisol to 5 $\beta$ -dihydrocortisol (**Figure 3D**). Studies with anaerobically purified OsrB  
157 revealed that NADH and NADPH were poor electron donors for OsrB. Using the artificial  
158 electron donor methyl viologen, we observed that OsrB similarly reduced a variety of steroid  
159 hormones substrates (**Figure 3E**). These results thus establish OsrB as a promiscuous 3-oxo-  
160  $\Delta$ 4-steroid hormone reductase that uses a presently unidentified electron donor.

161  
162 **Short chain dehydrogenase OsrC is a 3-oxo-5 $\beta$ -steroid hormone oxidoreductase**

163 We next sought to identify the *C. steroidoreducens* enzyme responsible for reduction  
164 of the 3-oxo group on the 5 $\beta$ -dihydrosteroid intermediate generated by OsrB. Microbial bile  
165 acid oxidoreductases with specificity for 3 $\alpha$ -, 3 $\beta$ -, 7 $\alpha$ -, 7 $\beta$ -, 12 $\alpha$ - and 12 $\beta$ -hydroxyl groups  
166 have been previously identified.<sup>32-34</sup> As these characterized steroid oxidoreductases are  
167 members of the short chain dehydrogenase (SDR) enzyme superfamily, we reasoned the *C.*  
168 *steroidoreducens* enzyme was likely related to this family. An analysis of the *C.*  
169 *steroidoreducens* genome identified 12 genes with SDR domains. However, none were  
170 induced by cortisol or exhibited high sequence similarity to previously characterized bile acid  
171 oxidoreductases.

172 As these analyses failed to identify obvious candidates, we next performed an  
173 unbiased screen of SDR-containing *C. steroidoreducens* proteins for 3-oxo-5 $\beta$ -steroid  
174 hormone reductase activity. We confirmed soluble expression of all 12 SDRs in *E. coli* and  
175 tested the activity of overexpressing *E. coli* strains on 5 $\beta$ -dihydrocortisol (**Figure 4A,**  
176 **Extended Data Figure 3**). We identified two SDRs (BLEONJ\_2554 and BLEONJ\_1088) that  
177 produced 3 $\beta$ ,5 $\beta$ -tetrahydrocortisol and two others (BLEONJ\_2478 and BLEONJ\_1414) that  
178 yielded 3 $\alpha$ ,5 $\beta$ -tetrahydrocortisol (**Figure 4A**).

179 Studies with anaerobically purified BLEONJ\_2554 and BLEONJ\_1088 revealed  
180 divergent substrate specificities. BLEONJ\_2554 showed a pronounced preference for 5 $\alpha$ -  
181 steroids and exhibited weak activity that did not follow classical Michaelis-Menten kinetics with  
182 5 $\beta$ -steroid substrates (**Figure 4B, Extended Data Figure 4**). Conversely, gene BLEONJ\_1088  
183 displayed a preference for 5 $\beta$ -steroid hormones and accommodated multiple functional groups  
184 at the C9 or C17 positions (**Figure 4B, Extended Data Figure 5**). We further found that  
185 BLEONJ\_1088 exhibited a preference for steroid hormones relative to the comparable bile acid  
186 derivative lithocholic acid. We thus conclude that BLEONJ\_1088 is a 3-oxo- $\Delta$ <sup>4</sup>-steroid hormone  
187 reductase and, on this basis, renamed it OsrC.

188  
189 **Fe-S flavoenzyme family OsrA is a 3-oxo- $\Delta$ <sup>1</sup>-steroid hormone reductase active on**  
190 **synthetic corticosteroids**

191 Synthetic corticosteroids possess potent anti-inflammatory properties and are used to  
192 treat a range of pathologies, including inflammatory bowel disease.<sup>35</sup> The synthetic  
193 corticosteroids drugs dexamethasone, prednisone, prednisolone, and methylprednisolone

194 contain a  $\Delta^1$ -bond that is absent in natural corticosteroids and which significantly extends their  
195 half-life (**Figure 5A**).<sup>36</sup> As our initial screen of steroids identified prednisolone as a substrate for  
196 *C. steroidoreducens* (**Figure 1**), we sought to address the molecular basis of synthetic  
197 corticosteroid metabolism. We first tested *C. steroidoreducens* activity on additional synthetic  
198 corticosteroids dexamethasone, prednisone, and methylprednisolone and found that all were  
199 reduced to  $3\beta,5\beta$ -tetrahydrocortisol derivatives, indicating that the bacterium possesses both  $\Delta^1$ -  
200 and  $\Delta^4$ -steroid hormone reductase activities (**Figure 5B**).

201 Considering the similarity of the  $\Delta^1$ -reduction to the OsrB catalyzed  $\Delta^4$ -reduction, we next  
202 tested the activity of OsrA and OsrB and found that the two enzymes generated distinct cortisol  
203 and testosterone isomers from prednisolone and the synthetic androgen boldenone,  
204 respectively (**Figure 5C, Extended Data Figure 6**). Based on comparison to reference  
205 standards, we establish that OsrA and OsrB products reflected  $\Delta^1$ - and  $\Delta^4$ -steroid hormone  
206 reductase activities, respectively (**Extended Data Figure 6**). These results demonstrate that  
207 OsrA functions as a  $\Delta^1$ -steroid hormone reductase that acts in conjunction with OsrB and OsrC  
208 to reduce synthetic steroid hormones to  $3\beta,5\beta$ -reduced products (**Figure 5E**).

### 209 **Steroid hormone reductase activities are common in gut bacteria and correlate with the** 210 **distribution of *osrA* and *osrB* homologs**

211 We next sought to address the breadth of steroid hormone reductase activity in the gut  
212 microbiome. We performed BLASTp searches of OsrA, OsrB, and OsrC in the Unified Human  
213 Gastrointestinal Genome catalog of representative genomes and metagenome-assembled  
214 genomes, which includes 4,644 prokaryotic species that colonize the human gastrointestinal  
215 tract.<sup>37</sup> These searches identified homologs with high sequence homology to OsrA, OsrB, and  
216 OsrC in 2, 59, and 90 genomes, respectively (**Supplementary Table 2**). Genomes encoding  
217 *osrABC* homologs included gram-positive bacterial species from multiple taxa, primarily from the  
218 Erysipelotrichaceae and Lachnospiraceae families.

219 To determine the association of *osrABC* homologs with observed *C. steroidoreducens*  
220 phenotypes, we selected 117 gut bacteria strains for experimental characterization. We tested  
221 these strains on solid media on steroid clearance/precipitate accumulation and assayed a select  
222 subset for cortisol and prednisolone activity. From these studies we identified 29 strains from 14  
223 species with a steroid clearance/precipitate accumulation phenotype and 6 species isolates with  
224 steroid hormone reductase activity (**Figure 6A-6C and Supplementary Table 3**).

225 Comparing strain genotypes to observed phenotypes revealed several patterns. First,  
226 presence of an *osrB* homolog in a genome strongly predicted steroid clearance/precipitate  
227 accumulation and steroid hormone  $\Delta^4$ -reductase activity (**Figure 6B and Supplementary Table**  
228 **3**). Second, the absence of *osrA* homologs tracked with a consistent lack of steroid hormone  $\Delta^1$ -  
229 reductase activity (**Figure 6C and Supplementary Table 3**). Third, while the presence of an  
230 *osrC* homolog tracked with production of  $3\beta,5\beta$ -tetrahydrocortisol, absence of an *osrC* homolog  
231 was not predictive of fate of the C3 functional group. Indeed, *osrC*-negative strains varied in  
232 their major cortisol product, generating either  $5\beta$ -dihydrocortisol,  $3\alpha,5\beta$ -tetrahydrocortisol, or  
233  $3\beta,5\beta$ -tetrahydrocortisol (**Figure 6B and Supplementary Table 3**). These results provide  
234 evidence that *osrA* and *osrB* specifically confer  $\Delta^1$ - and  $\Delta^4$ -steroid hormone reductase activities,  
235 respectively, while *osrC* likely represents one of multiple evolutionarily distinct 3-oxo- $5\beta$ -steroid  
236 hormone oxidoreductases.

### 237 ***osrB* is prevalent in human fecal metagenomes and associated with active Crohn's** 238 **disease**

239 Having established the relevance of *osrABC* homologs for steroid reductase activity in  
240 gut bacteria, we next sought to determine the prevalence of the pathway in the human gut. We  
241 focused our analysis on *osrA* and *osrB*, since these homologs reliably predicted steroid  
242 hormone reductase activities of assayed strains. We recruited reads to *osrA* and *osrB* homologs  
243  
244

245 in a collection of 1,491 previously published healthy human fecal metagenomes. We detected at  
246 least one read mapping to *osrA* and *osrB* homologs in 2.2% and >98.9% of samples,  
247 respectively (**Supplementary Table 4**). Within most metagenomes multiple *osrB* homologs  
248 recruited many reads. By contrast, the majority *osrA* reads recruited to *Clostridium tertium osrA*  
249 homologs, often with only one or two reads per metagenome (**Supplementary Table 4**). These  
250 analyses demonstrate that *osrB* homologs are common in the gut but that *osrA* homologs are  
251 confined to bacteria that colonize the gut at a low relative abundance.

252 Considering that glucocorticoids possess potent anti-inflammatory activities and natural  
253 and synthetic variants, including cortisol and prednisolone, are rectally and orally administered  
254 for the treatment of inflammatory bowel disease, we reasoned that OsrA and OsrB activity could  
255 be clinically relevant in this patient population. We analyzed 314 metagenomes from the Lewis  
256 et al.<sup>38</sup> study of active Crohn's disease patients, including a subset treated with corticosteroids  
257 (**Supplementary Table 5**). We observed *osrB* homologs were elevated in Crohn's disease  
258 patient relative to a healthy control population (**Figure 6D**). Further scrutiny revealed that the  
259 increased abundance *osrB* homologs from *Ruminococcus\_B gnavus* and *Clostridium\_AQ*  
260 *innocuum*, two taxa previously associated with Crohn's disease inflammation,<sup>39,40</sup> was the  
261 primary driver of this association (**Figure 6E**).

262 *osrA* homologs similarly exhibited elevated abundance in Crohn's disease  
263 metagenomes, but their low prevalence coupled with the relatively small sample size of this  
264 dataset complicated statistical analysis of the significance of this relationship (**Figure 6D**). To  
265 address this issue, we expanded our dataset to include 1537 metagenomes from multiple  
266 separate studies that included Crohn's disease and control populations. Analysis of this larger  
267 dataset confirmed that *osrA* homologs were significantly elevated in Crohn's disease patient  
268 metagenomes and revealed that the *osrA* from *Clostridium tertium* was the primary driver of this  
269 association (**Extended Data Figure 7A and 7B, Supplementary Table 5**).

270 To determine whether identified associations extended an independent dataset, we  
271 evaluated 569 Crohn's disease patient metagenomes collected as part of Integrative Human  
272 Microbiome Project (**Supplementary Table 6**). We observed a similar association between  
273 elevated abundance of *osrA* and *osrB* homologs and microbiome dysbiosis scores used as a  
274 proxy for active Crohn's disease in this study (**Extended Data Figure 8A-8C, Supplementary**  
275 **Table 6**).<sup>41</sup> Underscoring the relevance of these observations to active Crohn's disease,  
276 metagenomes from this study with microbiome dysbiosis score consistent with inactive Crohn's  
277 disease exhibited intermediate *osrB* homolog levels between dysbiotic Crohn's disease and  
278 control non-IBD populations (**Extended Data Figure 8A, Supplementary Table 6**).

279 We further investigated the relationship between microbial steroid reductases and  
280 corticosteroid treatment in Crohn's disease patients, as reported in the Lewis et al.<sup>38</sup> study. Our  
281 analysis revealed that corticosteroid therapy was associated with an increase in *osrA* homolog  
282 prevalence, as these homologs were detected in 9.6% of corticosteroid-treated patients  
283 compared to only 2.4% in untreated patients. This suggests a potential selective pressure  
284 favoring bacteria that metabolize synthetic corticosteroids in patients receiving these therapies.  
285 Interestingly, within the metagenomes where *osrA* homologs were present, their abundance did  
286 not significantly differ between treated and untreated patients, suggesting that the effect of  
287 corticosteroid treatment may relate to increased colonization of bacteria with *osrA* homologs  
288 (**Extended Data Figure 9**).

## 289 **DISCUSSION**

291 In this study, we characterize *Clostridium steroidoreducens* HCS.1, a previously  
292 uncharacterized gut bacterium that encodes a novel reductive pathway, OsrABC, for the  
293 metabolism of steroid hormones. Our work expands on previous studies of microbial steroid  
294 metabolism by demonstrating the widespread prevalence and activity of OsrB and OsrC in the  
295 gut microbiota, which catalyze the reduction of natural steroid hormones into their 3 $\beta$ 5 $\beta$ -

296 tetrahydro derivatives. Notably, the OsrB homologs are prevalent in Crohn's disease-associated  
297 bacterial communities, implicating these enzymes in both health and disease contexts.

298 One of the most compelling aspects of this work is the link between microbial steroid  
299 metabolism and chronic inflammatory conditions, particularly Crohn's disease. Our data indicate  
300 that *osrB* homologs are enriched in pro-inflammatory taxa such as *Clostridium\_AQ innocuum* and  
301 *Ruminococcus\_B gnavus*, both of which have previously been associated with Crohn's  
302 disease.<sup>39,40</sup> Pro-inflammatory gut microbes often exhibit a competitive advantage in  
303 inflammatory conditions and induce inflammation to generate conditions favorable for their  
304 growth.<sup>41</sup> This suggests that these microbes may leverage steroid hormone metabolism to gain  
305 a competitive advantage in the inflamed gut environment. Specifically, OsrB-mediated depletion  
306 of endogenous anti-inflammatory glucocorticoids may represent an adaptive strategy employed  
307 by *Clostridium\_AQ innocuum* and *Ruminococcus\_B gnavus* to perpetuate inflammation and  
308 support their growth.

309 In a clinical context, the OsrABC reductase pathway may also have significant  
310 implications for glucocorticoid therapies, which are commonly administered to manage  
311 inflammatory bowel disease. Our findings suggest that the OsrABC reductase pathway could  
312 modulate the effective dose of administered glucocorticoids by degrading these anti-  
313 inflammatory compounds. This underscores the importance of further investigations into the  
314 microbial impact on drug bioavailability in relation to both the efficacy and dosing of steroid  
315 therapies in patients.

316 Beyond the clinical considerations, our study highlights the broader significance of gut  
317 microbial steroid metabolism in human health. Notably, a concurrently published manuscript  
318 independently identifies the 3-oxo- $\Delta^4$ -steroid hormone reductase activity of OsrB homologs,  
319 along with the characterization of additional novel gut bacterial enzymes that metabolize  
320 progestins.<sup>42</sup> Together, these findings represent a crucial step forward in delineating the broader  
321 landscape of microbial steroid hormone metabolism and its potential clinical implications.

322  
323

## 324 **MATERIALS AND METHODS**

### 325 Steroid hormone enrichment culture

326 For each enrichment sample, 15 mM steroid hormone (cortisol, corticosterone,  
327 progesterone, or testosterone) suspensions were prepared in 1 mL basal growth medium (Difco  
328 M9 minimal salts, 20 mM acetate, 20 mM formate, tryptone 0.01% w/v, Bacto yeast extract  
329 0.01% w/v, trace vitamins and minerals, MgSO<sub>4</sub> 4.09% w/v; pH 6.5). Homogenized fecal  
330 samples were pelleted and washed 3x in phosphate buffer saline (PBS), then resuspended in 1  
331 mL saline. 20  $\mu$ L cell suspension was added to each enrichment culture condition and incubated  
332 for 72 hours. After 72 hours, 20  $\mu$ L of each culture was used to inoculate fresh media  
333 supplemented with its respective compound. Cultures were passaged a total of 4 times. After  
334 the final passage, a portion of each condition was preserved in 20% glycerol and frozen at -80  
335 °C. The remaining culture was pelleted and processed for 16S rRNA sequencing.

336

### 337 Isolation of HCS.1

338 Preserved stocks of enrichment culture samples were plated onto fresh brain heart  
339 infusion (BHI) agar and incubated for 4 days at 37 °C under anaerobic conditions (5% H<sub>2</sub>, 10%  
340 CO<sub>2</sub>, 85% N<sub>2</sub>). Distinct colonies were passaged to confirm purity and identified by 16S rRNA V4-  
341 V5 variable region sequencing. Purified isolates of HCS.1 were stored at -80 °C in a 20%  
342 glycerol suspension. Frozen glycerol stocks were deposited in the DFI Symbiotic Bacterial  
343 Strain Bank Repository (<https://dfi.cri.uchicago.edu/biobank/>).

344

### 345 Steroid clearance assay

346 To prepare steroid clearance assays, progesterone and cortisol amounts for a final  
347 concentration of 12 mM or 16 mM, respectively, were sterilized by suspension in 70% v/v  
348 ethanol, followed evaporation at room temperature for 2 hours. Dried steroid powders were  
349 sifted into autoclaved BHI agar and stirred rapidly, shortly before pouring into plates. Solid  
350 plates were stored under anaerobic conditions at 25 °C for at least 24 hours prior to use.

351 To test HCS.1 steroid clearance, solid BHI plates were incubated at 37 °C for 2 days.  
352 After 2 days, single colonies were picked and suspended in 200 µL PBS. Aliquots of the cell  
353 suspension were spread onto solid steroid plates and incubated anaerobically for 5 days at 37  
354 °C. To test steroid clearance of other bacterial strains, solid BHI plates were incubated at 37 °C  
355 for 4 days. After 4 days, single colonies from each isolate were picked and suspended in 200 µL  
356 PBS. 2 µL aliquots were spotted onto solid progesterone plates, dried, and incubated  
357 anaerobically for 3 days at 37 °C.

### 358 359 16S rRNA sequencing and analysis

360 Cells from final steroid enrichment passages were collected by centrifugation and their  
361 genomic DNA extracted using the QIAamp PowerFecal Pro DNA kit (Qiagen). Briefly, samples  
362 were suspended in a bead tube (Qiagen) along with lysis buffer and loaded on a bead mill  
363 homogenizer (Fisherbrand). Samples were then centrifuged, and the supernatant was  
364 resuspended in a reagent that effectively removed inhibitors. DNA was then purified routinely  
365 using a spin column filter membrane and quantified using Qubit. The 16S rRNA variable V4-V5  
366 region was amplified using universal bacterial primers, 564F and 926R. Amplicons were purified  
367 using magnetic beads, then quantified and pooled at equimolar concentrations. The Qiagen  
368 QIAseq one-step amplicon library kit was used to ligate Illumina sequencing-compatible  
369 adaptors onto amplicons. Reads were sequenced on an Illumina MiSeq platform to generate 2 x  
370 250 base pair reads, with 5,000-10,000 reads per sample. Amplified 16S rRNA amplicons were  
371 processed through the dada1 pipeline in R. Forward reads were trimmed at 210 bp and reverse  
372 reads were trimmed at 150 bp, to remove low-quality nucleotides. Chimeras were detected and  
373 removed using default parameters. Amplicon sequence variants between 300 and 360 bp in  
374 length were taxonomically assigned to the genus level using the RDP Classifier (v2.13) with a  
375 minimum bootstrap confidence score of 80.

### 376 377 Sample preparation for whole genome sequencing

378 To prepare HCS.1 for whole genome sequencing, 10 mL BHI broth was inoculated with  
379 cells from a single bacterial colony and incubated anaerobically at 37 °C for 48 hours. The  
380 culture was centrifuged at 4000 x g for 10 minutes. The resulting pellet was resuspended,  
381 washed in phosphate buffer saline (PBS), and re-centrifuged.

### 382 383 Whole genome sequencing library preparation: Illumina short reads

384 Samples for Illumina short sequencing were extracted using the QIAamp PowerFecal  
385 Pro DNA kit (Qiagen), as described in the preceding subsection. Libraries were prepared using  
386 200 ng of genomic DNA using the QIAseq FX DNA library kit (Qiagen). Briefly, DNA was  
387 fragmented enzymatically into shorter fragments and desired insert size was achieved by  
388 adjusting fragmentation conditions. Fragmented DNA was end repaired and 'A's' were added to  
389 the 3'ends to stage inserts for ligation. During ligation step, Illumina compatible Unique Dual  
390 Index (UDI) adapters were added to the inserts and prepared library was PCR amplified.  
391 Amplified libraries were cleaned up, and QC was performed using TapeStation 4200 (Agilent  
392 Technologies). Libraries were sequenced on an Illumina NextSeq 1000/2000 to generate  
393 2x150bp reads.

### 394 395 Whole genome sequencing library preparation: Oxford Nanopore long reads



396 Samples for Nanopore and Illumina hybrid assemblies were extracted using the high  
397 molecular weight NEB Monarch Genomic DNA Purification Kit. DNA was QC'ed using genomic  
398 Tapestation 4200. Nanopore libraries were prepared using the Rapid Sequencing Kit (SQK-  
399 RAD114) and sequenced on MinION R10.4.1 flow cells. Nanopore reads were base-called  
400 using ONT Guppy basecalling software version 6.5.7+ca6d6af, minimap2 version 2.24-r1122,  
401 and was demultiplexed using ONT Guppy barcoding software version 6.5.7+ca6d6af using local  
402 HPC GPU. N50 of the nanopore long read is 7077 base pairs, the average read length is 4529.4  
403 base pairs, while the average read quality is 15.6, which is typical of Nanopore reads. Hybrid  
404 assembly was performed with both nanopore and Illumina short reads using Unicycler  
405 v0.5.0.<sup>43,44</sup>

#### 407 Taxonomic classification of *Clostridium steroidoreducens* sp. nov. Strain HCS.1<sup>AT</sup>

408 The classification of strain HCS.1 as a novel species, *Clostridium steroidoreducens* sp.  
409 nov. was performed using GTDB-Tk (version 2.3.2)<sup>45</sup> on the KBase platform. Genome quality  
410 assessment, phylogenetic placement, and taxonomic classification were performed according to  
411 GTDB guidelines. The HCS.1 genome was uploaded to the KBase website and analyzed using  
412 the GTDB-Tk classify workflow, which assigns genomes to the closest known taxa based on  
413 conserved marker genes. Strain HCS.1 was classified within the genus *Clostridium*, but did not  
414 match any known species in the Genome Taxonomy Database (GTDB, version r207).  
415 Phylogenetic placement within the GTDB bacterial tree confirmed that the strain represented a  
416 distinct lineage, supporting its designation as a new species.

#### 418 Transcriptomic analysis of HCS.1

419 To prepare HCS.1 samples for transcriptomic analysis, six 50 mL Lysogeny broth  
420 cultures were inoculated with bacteria cells and shaken anaerobically at 37 °C for 48 hours. After  
421 48 hours, cortisol powder was added to 3 cultures, to a final concentration of 8 mM. All 6  
422 cultures were incubated for an additional 4 hours, then pelleted at 4000 x g for 10 minutes. The  
423 resulting pellets were flash-frozen in a dry ice/ethanol bath and stored at -80 °C until ready for  
424 subsequent processing. Cell pellets were thawed and total RNA from biological replicates  
425 extracted using the Maxwell RSC instrument (Promega). Extracted RNA was quantified using  
426 Qubit, and integrity was measured using TapeStation (Agilent Technologies). Libraries from ribo-  
427 depleted samples were constructed using the NEB's Ultra Directional RNA library prep kit for  
428 Illumina. First, up to 500 ng total RNA was subjected to ribosomal RNA depletion (for bacteria)  
429 using NEBNext rRNA depletion kit. Ribosomal -RNA depleted samples were fragmented based  
430 on RNA integrity number (RIN). Post cDNA synthesis, Illumina compatible adapters were ligated  
431 onto the inserts and final libraries were QC'ed using TapeStation (Agilent technologies).  
432 Libraries were normalized using library size and final library concentration (as determined by  
433 Qubit). Library concentration (ng/ul) was converted to nM to calculate dsDNA library  
434 concentration. Equimolar libraries were then pooled together at identical volumes to ensure  
435 even read distribution across all samples. Normalized libraries were then sequenced on  
436 Illumina's NextSeq 1000/2000 at 2x100bp read length.

437 High-quality reads were mapped to the circularized hybrid assembled genome of HCS.1  
438 (NCBI: CP170704), using Bowtie2 (v.2.4.5), and sorted with Samtools (v1.6). Read counts were  
439 generated using featureCounts (v2.0.1) with Bakta annotations.<sup>46</sup> Gene expression was  
440 quantified as the total number of reads uniquely aligning to the reference genome, binned by  
441 annotated gene coordinates. Differential gene expression and quality control analyses were  
442 performed using DESeq2 in R with Benjamini-Hochberg false discovery rate adjustment applied  
443 for multiple testing corrections.<sup>47</sup>

#### 445 Bacterial culture steroid reductase assay

446 A complete list of strains used in this study is provided in **Supplementary Table 2**.  
447 Strains were incubated under anaerobic conditions (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub>) at 37 °C in an  
448 anaerobic chamber (Coy Laboratory). Liquid brain-heart infusion (BHI) broth supplemented with  
449 100 µM steroids from 10 mM stocks in methanol was used for growth. Cultures were grown  
450 anaerobically with a 1% (v/v) inoculum from a pre-culture and supplemented with steroids after  
451 4 hours of incubation during the exponential growth phase. Bacterial cultures were extracted by  
452 the addition of 9 volumes of methanol supplemented with 0.5 µM methylprednisolone as an  
453 internal standard for LC-MS analysis.

#### 454 455 LC-MS-Q-TOF analysis of steroids

456 Extracted samples were vortexed and centrifuged twice at 21,000 × g for 15 minutes,  
457 with the supernatant transferred to new tubes after each centrifugation step. The methanol  
458 fraction was filtered through 0.2 µm nylon membrane filters prior to LC-MS analysis. Samples  
459 were analyzed using an Agilent 6540 UHD Q-TOF mass spectrometer coupled to an Agilent  
460 1200 Infinity LC system. Separation was performed on a XBridge C18 column (2.1x100mm, 3.5  
461 µm particle size) using 0.1% aqueous formic acid and acetonitrile with 0.1% formic acid as  
462 mobile phases. The separation gradient ranged from 20% to 100% acetonitrile over 4 minutes at  
463 50 °C with a flow rate of 0.5 mL/min. Mass spectra were acquired in negative ion mode for  
464 glucocorticoids ([M+FA-H]<sup>-</sup>) or positive ion mode for all other steroids ([M+H]<sup>+</sup>), with an ion spray  
465 voltage of 3500 V and a nozzle voltage of 2000 V. The source temperature was set to 300 °C,  
466 and the gas flow rate was 8 L/min. Data were processed and visualized using MassHunter  
467 software version 10.

#### 468 469 Molecular biology

470 Gene transformations were performed by Gibson assembly using 2x NEBuilder® HiFi  
471 DNA Assembly Master Mix (New England Biolabs, NEB, E2621X). Primers were designed using  
472 SnapGene (see **Supplementary Table 7**), incorporating 20 bp flanking regions complementary  
473 to a linearized expression vector (pMCSG53) and the gene of interest from the HCS.1 genome.  
474 PCR, cloning, and transformation were performed according to the protocols provided on the  
475 NEB website. The Gibson assembly reaction was incubated at 50 °C for 1 hour and then  
476 transformed into *E. coli* XL1-Blue competent cells according to the manufacturer's protocol.  
477 Transformed cells were plated on Luria-Bertani (LB) agar plates containing 100 µg/mL  
478 carbenicillin, and successful transformations were confirmed using sequencing primers specific  
479 for the backbone vector (see **Supplementary Table 7**). Positive colonies were validated and  
480 sequenced by the University of Chicago Genomics Facility. The final constructs were then  
481 transformed into chemically competent *E. coli* Rosetta™ (DE3) competent cells (Novagen)  
482 according to NEB protocols. Transformed cells were plated on LB agar plates supplemented  
483 with 100 µg/mL carbenicillin.

#### 484 485 Protein production in *E. coli*

486 Protein production in *E. coli* Rosetta cells was performed under aerobic conditions for all  
487 short-chain dehydrogenases (SDRs) and under anaerobic conditions for OsrA and OsrB.  
488 Cultures were grown in either 2x YT medium (20 g/L tryptone, 10 g/L yeast extract, and 5 g/L  
489 NaCl) or TB medium (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 9.4 g/L K<sub>2</sub>HPO<sub>4</sub>,  
490 and 2.2 g/L KH<sub>2</sub>PO<sub>4</sub>) supplemented with 0.5% (w/v) glucose and 1 mM ferric ammonium citrate,  
491 respectively.

492 Induction of protein expression was initiated during the exponential phase when optical  
493 densities (OD<sub>600</sub>) reached 0.4-0.6 by the addition of 1 mM isopropyl β-D-1-  
494 thiogalactopyranoside (IPTG). Cultures were incubated for 3-5 hours at 37 °C with shaking at  
495 200 rpm. Cells were harvested by centrifugation at 4500 × g for 20 minutes. Cell pellets were

496 then frozen at -80 °C for storage prior to subsequent experimental use. Protein production was  
497 confirmed by SDS-PAGE analysis.

498

#### 499 Purification of heterologous produced proteins

500 *Cell Lysis and Protein Purification.* Frozen cell pellets were supplemented with 0.1 mg/mL  
501 DNase and lysed either aerobically (for SDRs) or anaerobically (for OsrA and OsrB) using a  
502 Thermo Spectronic French pressure cell at 1,100 PSI. The crude cell extract was centrifuged at  
503 75,600 x g for 30 minutes, followed by filtration through a 0.2 µm nylon membrane (Fisher  
504 Scientific) before being applied to a purification system.

505

506 *Aerobic purification of SDRs.* The filtered extract was applied to an ÄKTA pure system (Cytiva)  
507 using a 1 mL Strep-Tactin®XT 4Flow® column (Iba Lifesciences). The column was equilibrated  
508 with 10 volumes of equilibration buffer (100 mM Tris/HCl, pH 8.0, 150 mM NaCl) at 1 mL/min  
509 and 4°C. Proteins were loaded via a 5 mL loop, followed by washing of non-specifically bound  
510 proteins. Elution was performed with 5 mL elution buffer (100 mM Tris/HCl, pH 8.0, 150 mM  
511 NaCl, and 50 mM biotin). Eluted proteins were collected in 1 mL fractions and were  
512 concentrated using Pierce™ Protein concentrators (10 kDa), desalted, and either used directly  
513 or transferred to storage buffer (50 mM Tris/HCl, pH 7.5, 10% (w/v) glycerol, and 50 mM NaCl)  
514 using PD-10 desalting columns (Cytiva) before storage at -80 °C.

515

516 *Anaerobic purification of OsrB.* Anaerobic purification was performed in an anaerobic chamber.  
517 A Strep-Tactin®XT 4Flow® gravity column (Iba Lifesciences) was used with a WET FRED  
518 system (Iba Lifesciences) to maintain a constant flow rate of ~1 mL/min, adjusted using a lab  
519 jack stand (LABALPHA). The column was equilibrated with anaerobic equilibration buffer (100  
520 mM Tris/HCl, pH 8.0, 150 mM NaCl) and elution was performed with anaerobic elution buffer  
521 (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, and 50 mM biotin). Eluted proteins were collected in 1  
522 mL tubes, concentrated and desalted using Pierce™ Protein Concentrators PES, 10K MWCO,  
523 0.5 mL, at 10,000 x g in a microcentrifuge. Proteins were either used directly for enzymatic  
524 assays or transferred to anaerobic storage buffer (50 mM Tris/HCl, pH 7.5, 10% (w/v) glycerol,  
525 and 50 mM NaCl) and frozen at -80 °C.

526

#### 527 Whole-cell assays of heterologous enzymes

528 The activity of heterologously expressed proteins was assessed under either aerobic  
529 conditions (for SDRs) or anaerobic conditions (for OsrA and OsrB). *E. coli* Rosetta cells were  
530 grown in media as described above, supplemented with 100 µM steroids (prepared from 10 mM  
531 stock solutions in methanol) using a 1% (v/v) inoculum. Protein production was induced with 1  
532 mM IPTG at an OD of 0.4-0.7 and cultures were incubated overnight at 37 °C without shaking.  
533 Reactions were quenched by the addition of 9 volumes of methanol containing 0.5 µM  
534 methylprednisolone as internal standard (IS). LC-MS samples were prepared as described  
535 previously.

536

#### 537 Enzymatic assays with purified SDRs

538 The kinetic properties of purified SDRs were determined using reaction mixtures in 96-  
539 well plates with a total volume of 100 µL. The reaction mixture contained 25 mM Tris/HCl (pH  
540 7.0), 1 mM NADPH, 10 µM to 1 mM steroids (diluted in 20 mM hydroxypropyl-β-cyclodextrin),  
541 and 0.01 to 0.5 mg/mL protein, depending on the enzyme activity. Enzyme activity was  
542 monitored by measuring the reduction of NADPH at 340 nm using a plate reader (BioTek,  
543 Cytation 5) at 37°C. A NADPH standard curve was analyzed under identical conditions with an  
544 extinction coefficient of 1398 M<sup>-1</sup> for quantitation.

545

#### 546 Enzymatic assays with purified OsrA and OsrB

547 The substrate preferences of OsrA and OsrB were analyzed under anaerobic conditions  
548 using a 100  $\mu$ L reaction mixture containing 50 mM Tris/HCl (pH 7.0), 200  $\mu$ M methyl viologen,  
549 0.5 mM steroids (from 10x stock solutions in methanol), and 50  $\mu$ g/mL protein. Enzyme activity  
550 was monitored by measuring electron donor reduction at 605 nm using a plate reader (BioTek,  
551 Epoch 2) at 37°C. Quantification was performed using an electron donor standard curve  
552 generated under the same conditions with an extinction coefficient of 1689 M<sup>-1</sup> for quantitation.  
553

#### 554 Phylogenetic tree construction

555 Genome metadata were retrieved from a local UHGG database and used to map  
556 genome IDs to species names. A comparative analysis was performed using BLASTp  
557 (version 2.15.0+) to search for homologs of the target sequence against the UHGP-100  
558 database, limiting results to the top 20000 hits. The BLASTp output was processed to map  
559 genome IDs to species names and format the sequences in FASTA format, removing  
560 duplicates to ensure data quality. Additional sequences were appended to the data set as  
561 needed. Sequence alignment was performed using Clustal Omega (version 1.2.2)<sup>48</sup> with  
562 output formatted as FASTA. Header sanitization was performed to remove special characters,  
563 and duplicate sequences were filtered out using custom Python scripts to maintain alignment  
564 integrity. Phylogenetic analysis was performed using IQ-TREE (version 2.3.6)<sup>49</sup> with  
565 automatic model selection to determine the best-fitting substitution model based on the data.  
566 The reliability of the phylogenetic trees was assessed using 1,000 ultrafast bootstrap  
567 replicates to assess branch support. The final phylogenetic trees were visualized and  
568 interpreted using the Interactive Tree of Life (iTOL)<sup>50</sup> to explore the evolutionary relationships  
569 among the identified protein sequences.  
570

#### 571 Metagenomics

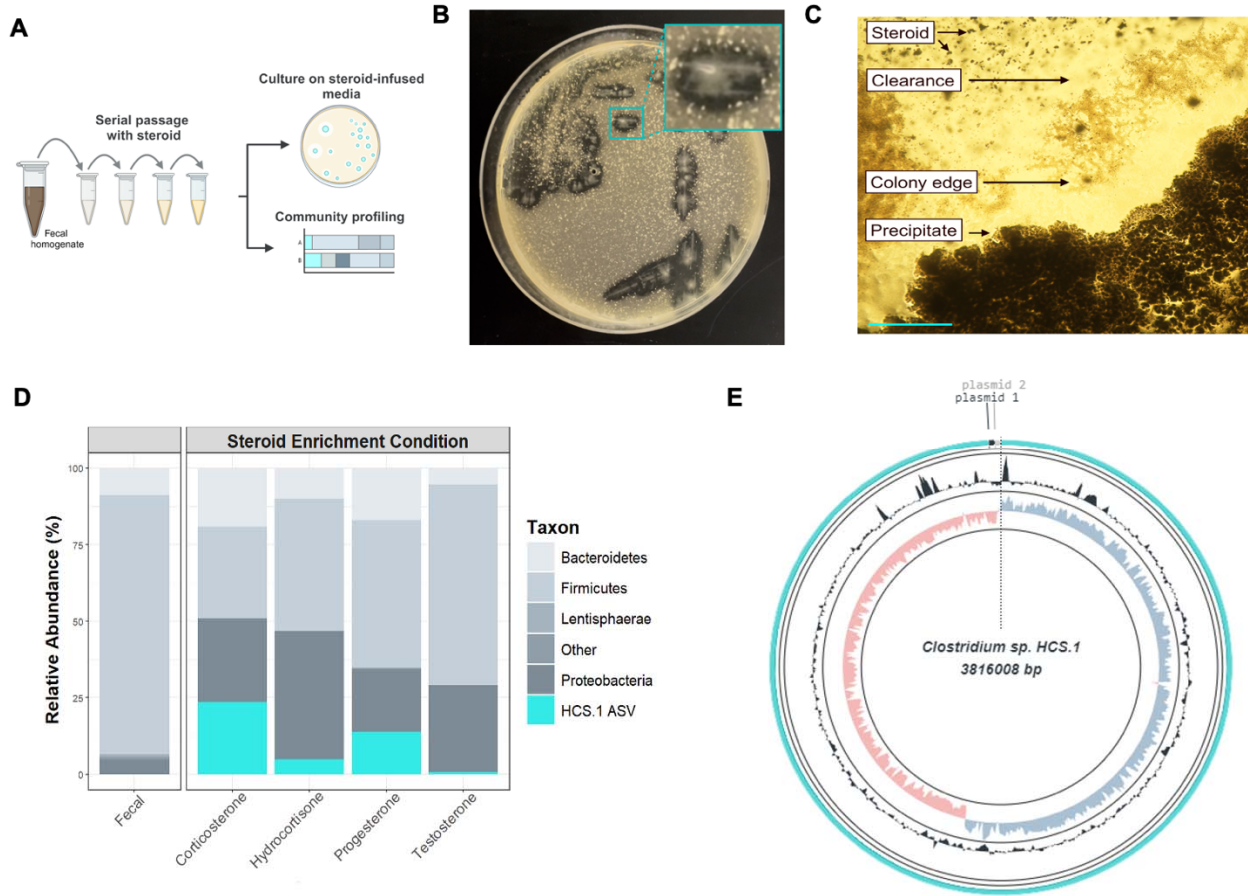
572 To identify relevant sequences of OsrA or OsrB, a BLAST search was first conducted  
573 against the UHGP-100 database, applying a 49% similarity threshold based on experimental  
574 evidence indicating that this threshold effectively identifies relevant homologs while minimizing  
575 false positives. For OsrB, the identified protein sequences were used to construct a  
576 phylogenetic tree using Clustal Omega for alignment and IQ-TREE with the "mtest" model  
577 selection and 1,000 bootstrap replicates as described above. Based on the initial analysis, 25  
578 sequences that were not phylogenetically related to OsrB were manually excluded. This  
579 curation step ensured that only sequences relevant to the target enzymes were retained for  
580 downstream analysis.

581 After phylogenetic filtering, genome information was traced back using the UHGP protein  
582 IDs. The corresponding genomes were downloaded from the Unified Human Gastrointestinal  
583 Genome (UHGG) database using FTP links provided in the metadata file. The genomes were  
584 then used for further analysis, where each protein sequence was screened against the  
585 respective genome using tblastn with a stringent e-value threshold of 1e<sup>-200</sup> to ensure high  
586 specificity. The best nucleotide sequence was selected for each protein based on coverage and  
587 bit score and subsequently compiled into a combined FASTA file.

588 Metagenomic samples were downloaded from the Sequence Read Archive (SRA). Reads were  
589 quality trimmed to remove adapter sequences using TrimGalore with default settings,<sup>51</sup> and  
590 potential human contamination was removed by mapping the reads to the human reference  
591 genome (T2T-CHM13v2.0) using Bowtie2 (version 2.5.3) and removing the mapped reads with  
592 Samtools (version 1.61.1).<sup>52,53</sup> Samples were then mapped to the gene reference datasets for  
593 *osrA* and *osrB* using Bowtie2 (version 2.5.3), and copies per million (CPM values) were  
594 calculated for each gene in each sample. Samples with total read counts below 1,000,000 were  
595 excluded.

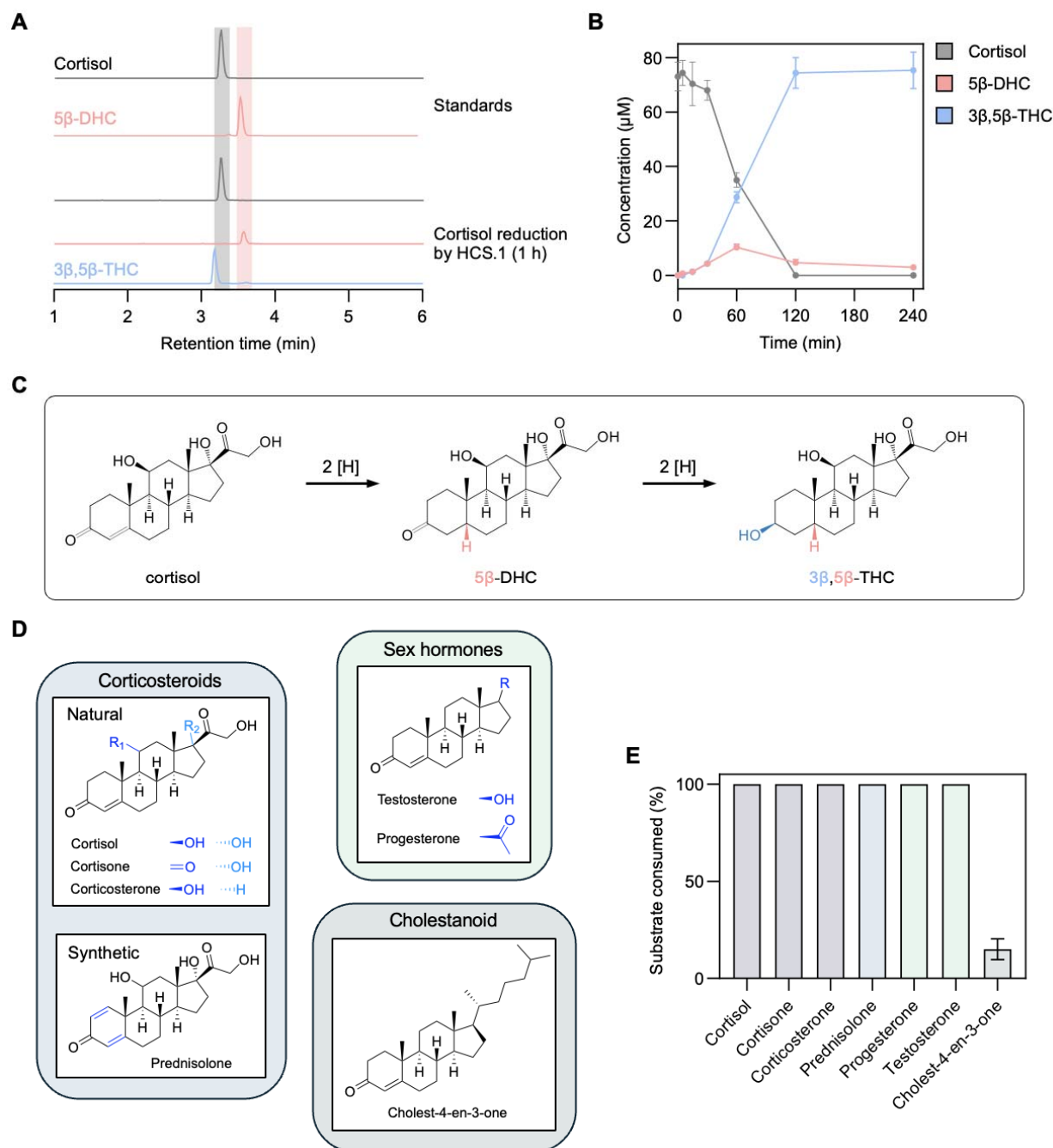
596 Metagenomic data were filtered to ensure quality for statistical analyses. Zero values  
597 were replaced with 1e<sup>-6</sup> for statistical assessment. A 99th percentile filter was applied to CPM

598 values for each gene (*osrA* and *osrB*) to remove extreme outliers. Normality was assessed  
599 using the Shapiro-Wilk test; if both groups were normal ( $p > 0.05$ ), a two-sided Welch's t-test  
600 was used to determine if there was any significant difference between the groups, regardless of  
601 direction. If normality was not met, a two-sided Mann-Whitney U test was applied. This  
602 conservative approach ensured that differences were detected without assuming the direction of  
603 the effect, providing flexibility in hypothesis testing. Analyses were performed using Python with  
604 Scipy, Pandas, and Seaborn libraries.



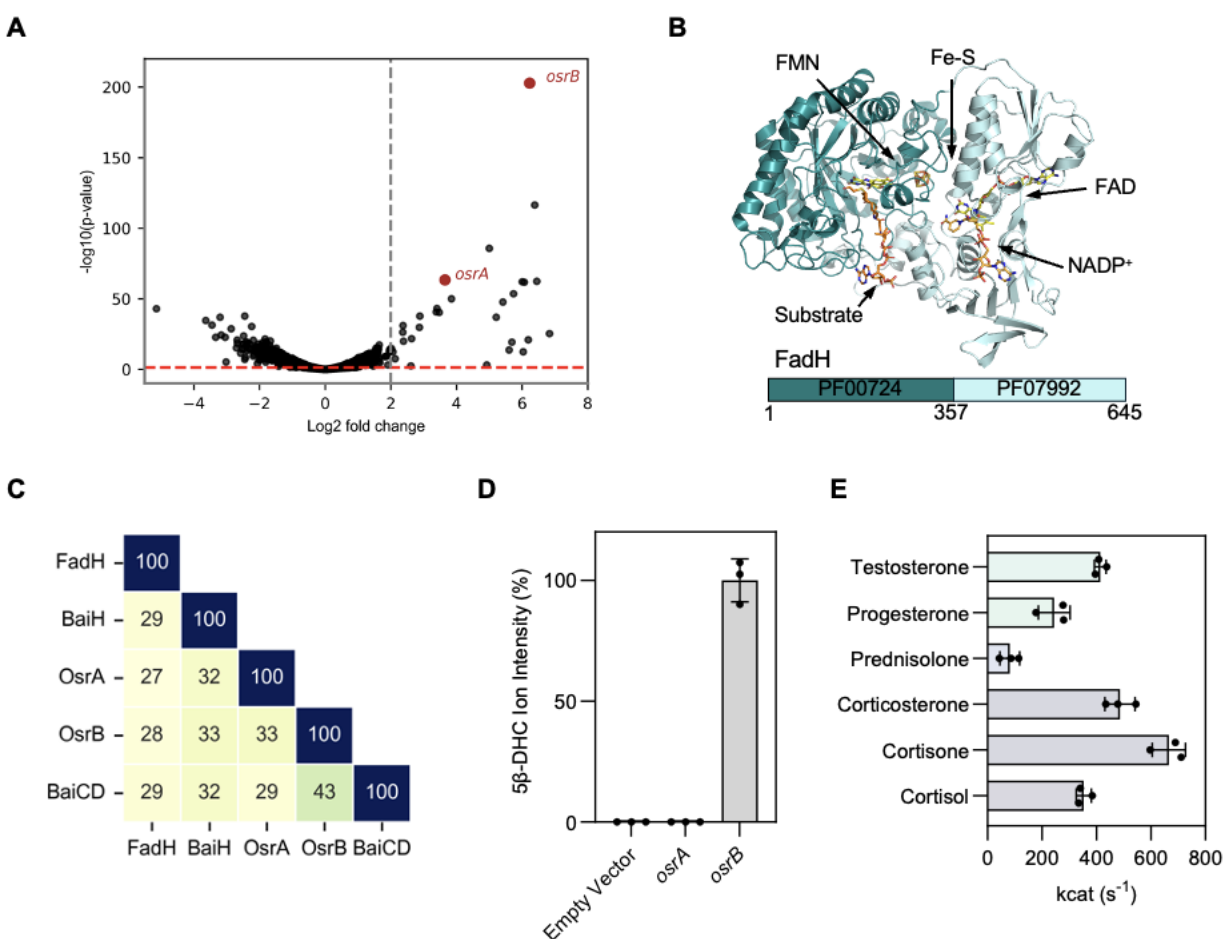
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**Figure 1. *Clostridium steroidoreducens* is a novel steroid-enriched species.** (A) Schematic overview of steroid enrichment and strain isolation experiments. (B) HCS.1 strain colonies on cortisol-infused media. (C) HCS.1 strain colonies on progesterone-infused media. Scale bar, 200 µm. (D) Microbial community profile of the final steroid hormone enrichment passage based on 16S rRNA amplicon sequencing. (E) Circular representation of the HCS.1 genome.



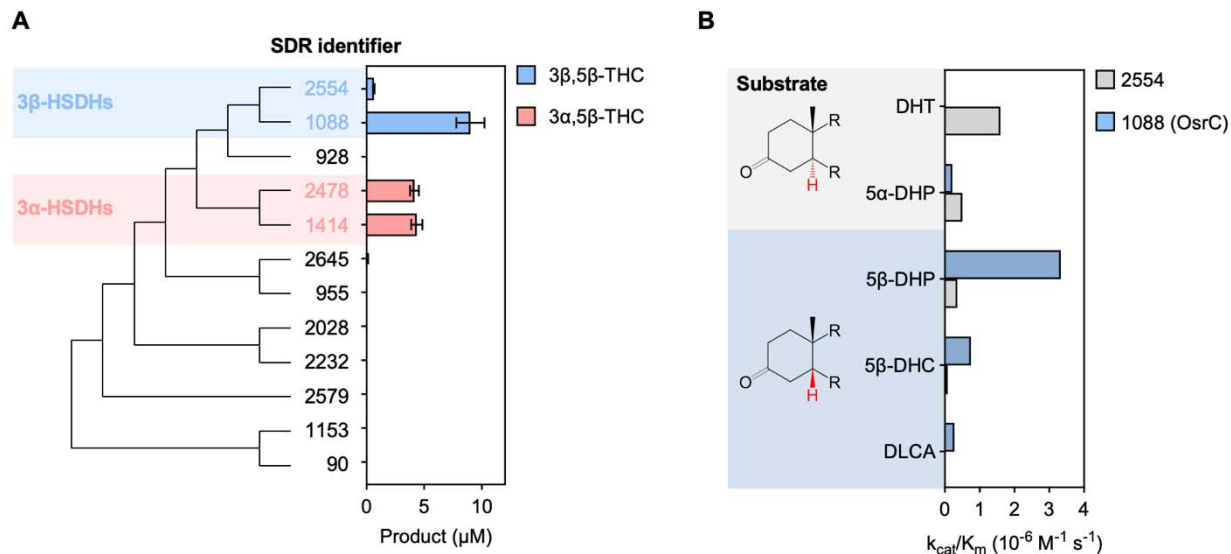
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**Figure 2. *C. steroidoreducens* HCS.1 possesses promiscuous 3-oxo- $\Delta^4$ -beta steroid hormone reductase activity.** (A) Products formed from *C. steroidoreducens* HCS.1 incubation with cortisol. (B) Time-course analysis of cortisol metabolism by *C. steroidoreducens* HCS.1. (C) Proposed pathway for cortisol reduction by *C. steroidoreducens* HCS.1. DHC and THC stand for dihydrocortisol and tetrahydrocortisol, respectively. (D) Steroid substrates tested for *C. steroidoreducens* HCS.1. (E) Measured *C. steroidoreducens* HCS.1 steroid substrate consumption.



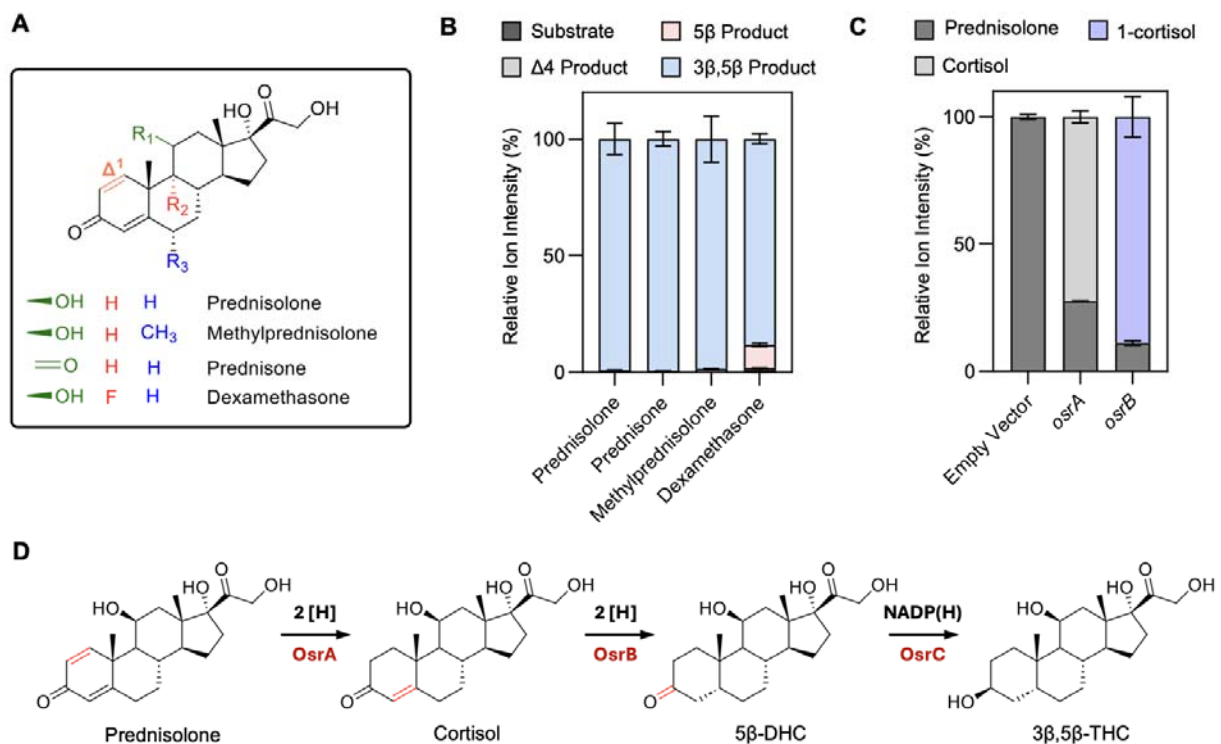
620  
 621 **Figure 3. OsrB is a 3-oxo- $\Delta^4$ -steroid hormone reductase.** (A) Gene expression of *C. steroidoreducens*  
 622 HCS.1 in the presence versus absence of cortisol. Gray and red dashed lines indicate genes with  
 623 statistical significance and >2-fold induction in response to cortisol, respectively. (B) Crystal structure of  
 624 Fe-S flavoenzyme 2,4-dienoyl-CoA reductase (FadH) bound to ligands (PDB code: 1PS9). (C) Percent  
 625 sequence identity of OsrA and OsrB to Fe-S flavoenzymes FadH and bile acid reductases BaiH and  
 626 BaiCD. (D) Conversion of cortisol to 5 $\beta$ -dihydrocortisol (DHC) by *E. coli* expressing *osrA* or *osrB* versus  
 627 an empty vector control. (E) Rate of reduction of indicated steroid hormones by purified OsrB.





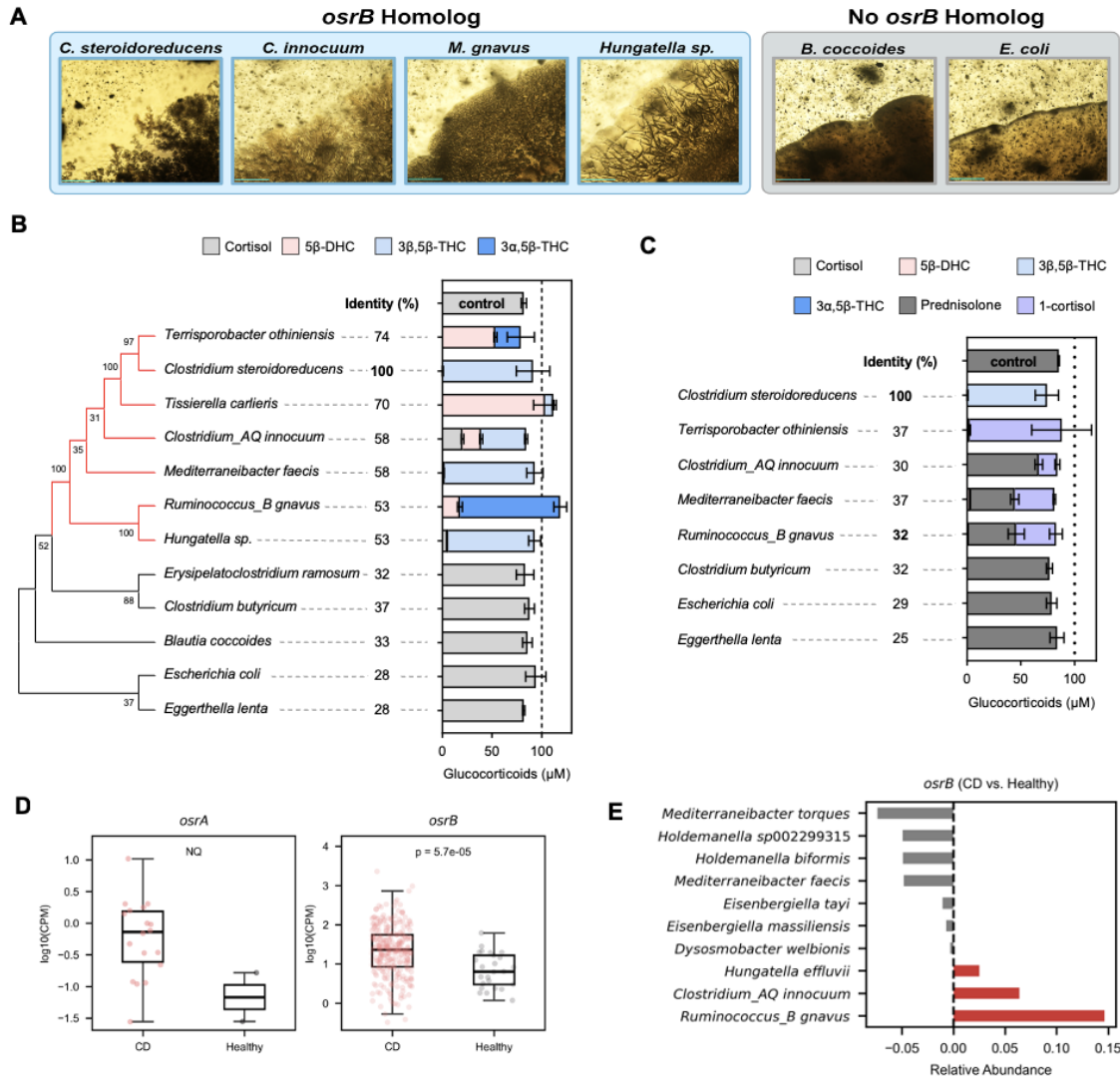
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**Figure 4. OsrC is a 3-oxo-5β-steroid hormone oxidoreductase.** (A) Phylogenetic analysis of *C. steroidoreducens* HCS.1 SDR domain-containing protein sequences. Product formed from 5β-dihydrocortisol by *E. coli* strains overexpressing SDR domain-containing proteins are shown with their respective gene identifiers. THC stand for tetrahydrocortisol. (B) Kinetic parameters of reduction of indicated steroid hormones by purified SDR domain-containing proteins. Abbreviations stand for dihydrotestosterone (DHT), dihydroprogesterone (DHP), dihydrocortisol (DHC), and dehydrolithocholic acid (DLCA).



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**Figure 5. OsrA is a 3-oxo- $\Delta^1$ -reductase essential for complete reduction of synthetic corticosteroids.** (A) Structure of synthetic corticosteroids used in assays. (B) Products formed from synthetic corticosteroids following incubation with *C. steroidoreducens* HCS.1 cells. (C) Percent prednisolone conversion to cortisol following incubation of *E. coli* cells with *osrA*- and *osrB*-expressing plasmids or an empty vector control. (D) *C. steroidoreducens* HCS.1 steroid reduction pathway identified in this study.



645  
646 **Figure 6. Steroid reductase activities are widespread in gut bacteria and elevated in active Crohn's**  
647 **disease.** (A) Representative images of gut bacteria grown on progesterone-infused media, showing  
648 steroid clearance/precipitate accumulation-positive (blue background) and -negative (gray background)  
649 colonies. Scale bar, 200 μm. (B) Corticosteroids produced by gut bacterial isolates after incubation with  
650 cortisol. The protein with the highest sequence identity to *OsrB* encoded by each genome was used to  
651 generate the tree. (C) Corticosteroids produced by gut bacterial isolates after incubation with  
652 prednisolone. Identity refers to the sequence identity of the protein with the highest sequence identity to  
653 *OsrA* encoded by each genome. (D) reads mapping to *osrA* and *osrB* homologs in metagenomes from  
654 healthy and Crohn's disease (CD) patients. Only metagenomes with at least one read mapping to a gene  
655 are included in the analysis. NQ refers to the not quantified statistical difference, due to the low number of  
656 healthy metagenomes with reads mapping to *osrA*. CPM refers to copies per million. (E) Difference in  
657 *osrB* homolog abundance for taxa showing the greatest changes in relative abundance between healthy  
658 and CD metagenomes.

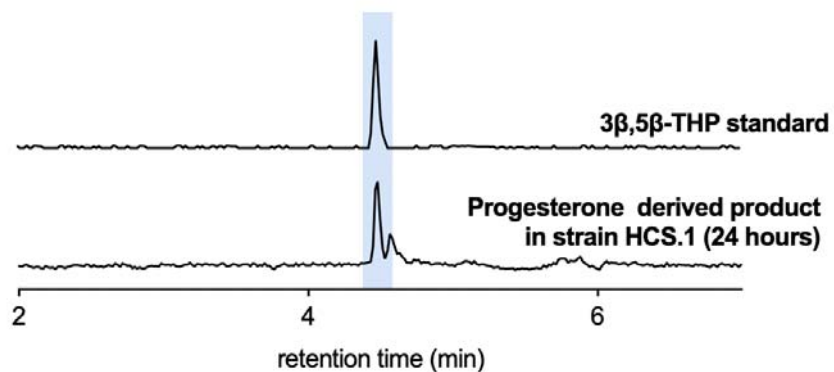
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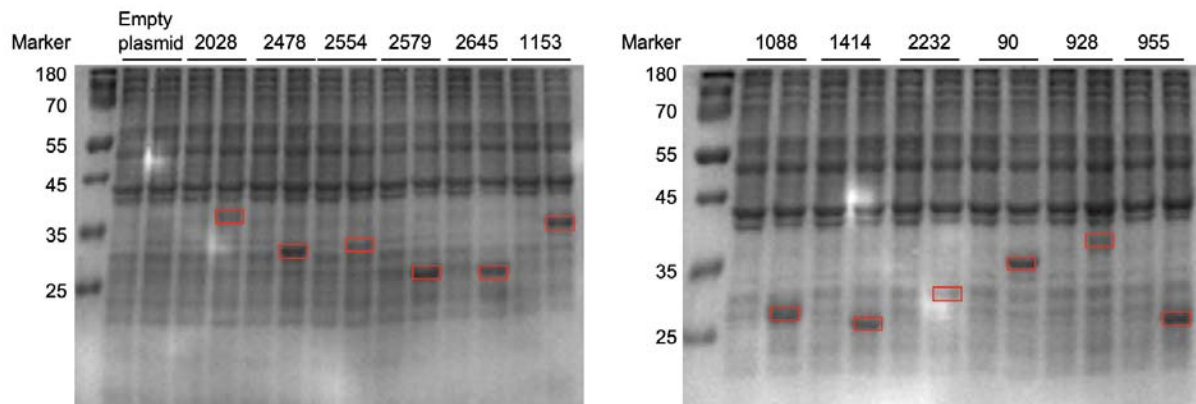




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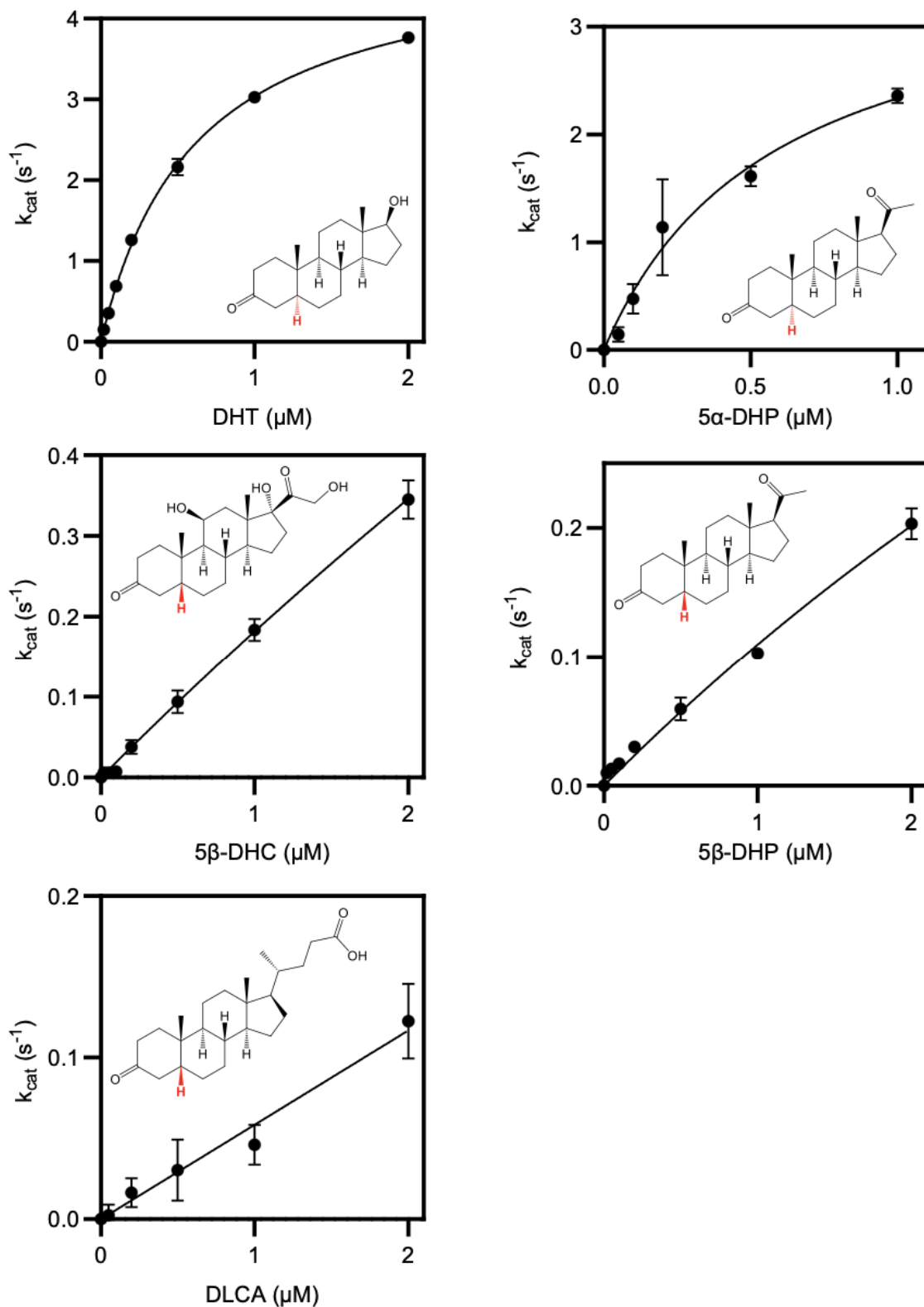
**Extended Data Figure 2. Product of *C. steroidoreducens* HCS.1 incubation with progesterone.**  
Major progesterone product formed by *C. steroidoreducens* HCS.1. Comparison with reference standards confirms 3β-,5β-tetrahydroprogesterone (THP) production.





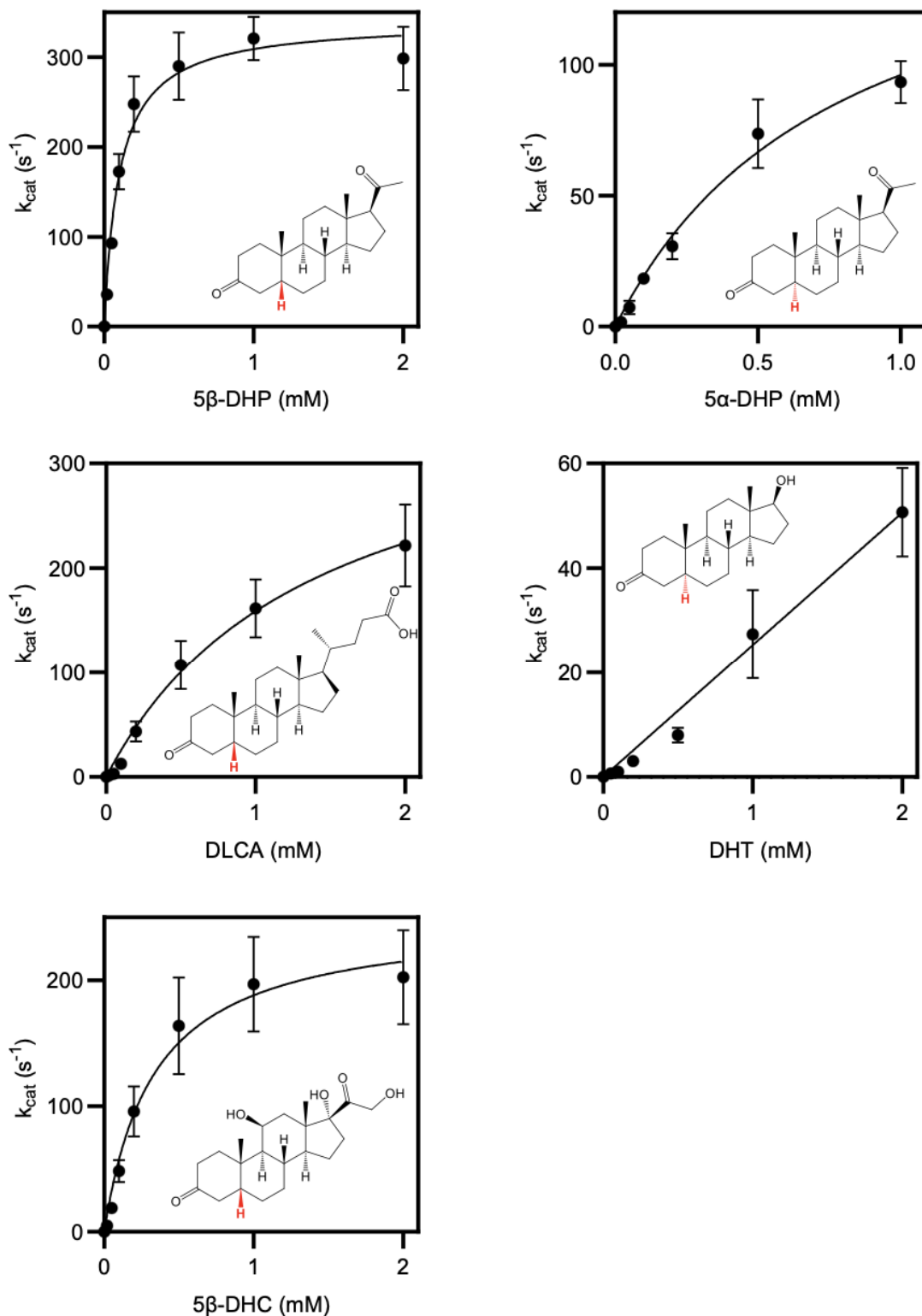
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**Extended Data Figure 3. 12% SDS-PAGE of *E. coli* lysates expressing SDR domain-containing proteins.** SDS-PAGE analysis showing the heterologous production of *C. steroidoreducens* SDR domain-containing proteins in *E. coli*, pre- and post-isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) induction. Red boxes highlight expressed protein.

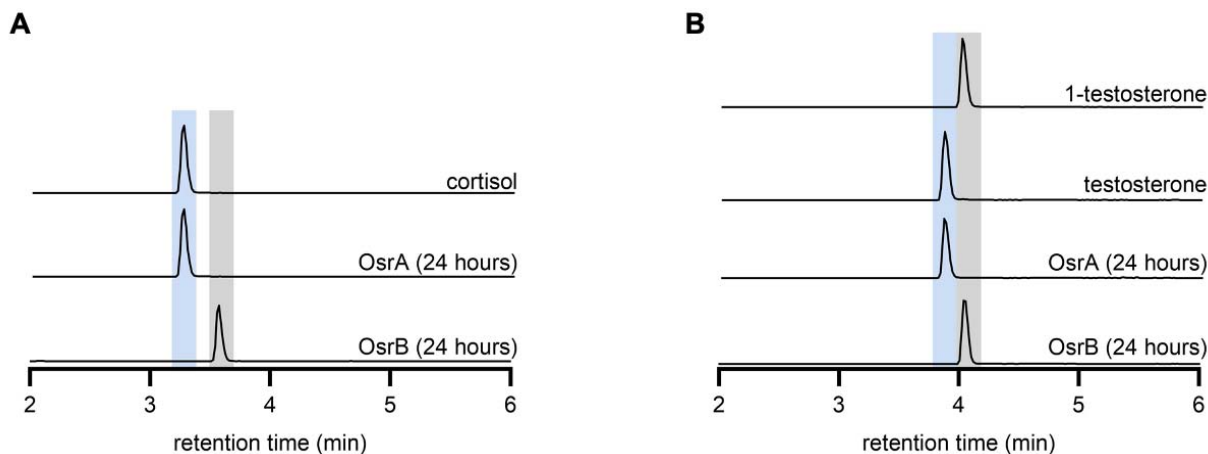


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**Extended Data Figure 4. Reaction rates of enriched BLEONJ\_2554 on indicated substrates.** Reaction rates for the 3'-reduction of 5-reduced steroids by anaerobically purified short-chain dehydrogenase BLEONJ\_2554.

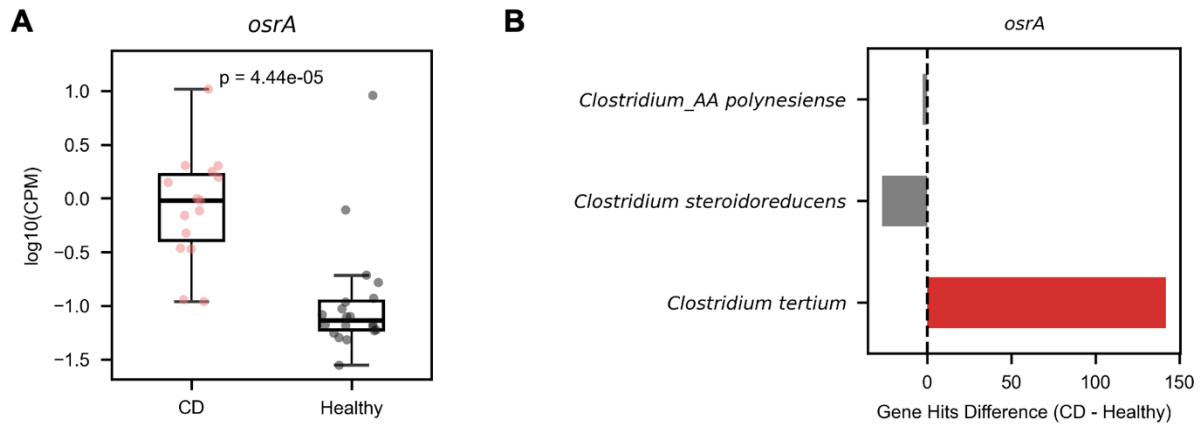


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801 **Extended Data Figure 5. Reaction rates of enriched OsrC on indicated substrates.** Reaction rates  
802 for the 3'-reduction of 5-reduced steroids by anaerobically purified short-chain dehydrogenase OsrC.



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804 **Extended Data Figure 6. OsrA and OsrB products from  $\Delta^1$ - and  $\Delta^4$ -steroid hormone substrates.** (A)  
805 Products following prednisolone incubation with purified OsrA or OsrB. Comparison with cortisol reference  
806 standard confirm that OsrA generates the  $\Delta^1$ -reduced product and show that OsrB produces a distinct  
807 cortisol isomer. (B) Products following boldenone incubation with purified OsrA or OsrB. Comparison to  
808 reference standards confirm that OsrA and OsrB generate  $\Delta^1$ - and  $\Delta^4$ -reduced products, respectively.

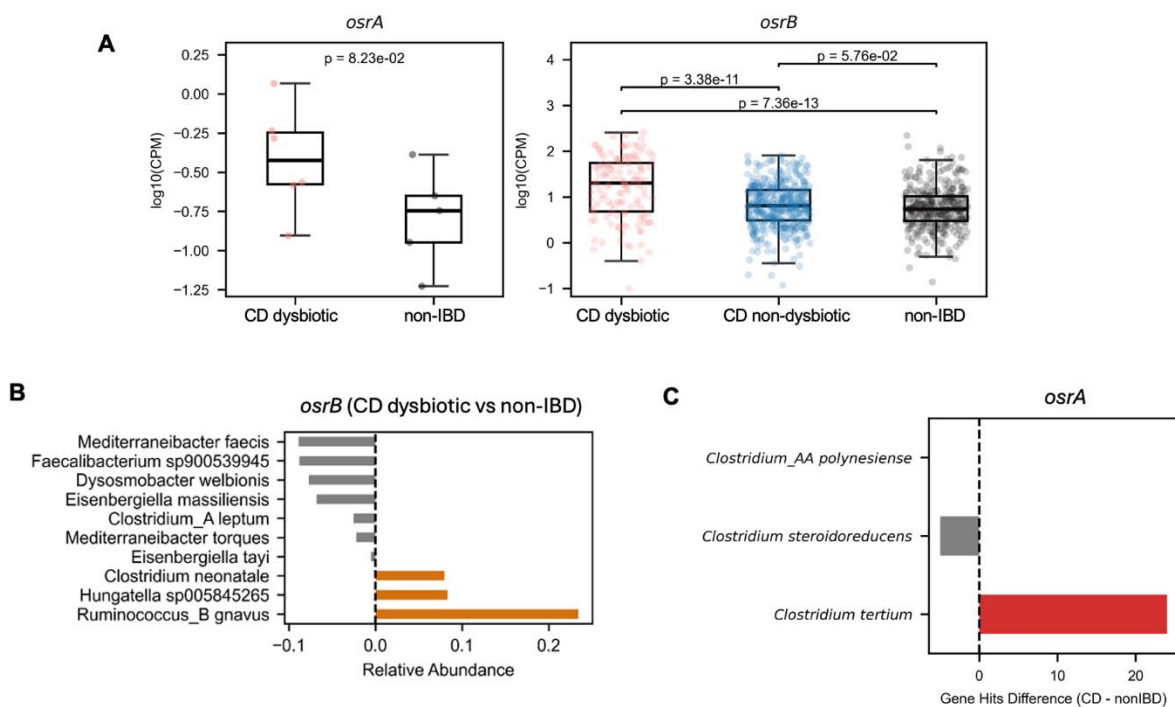
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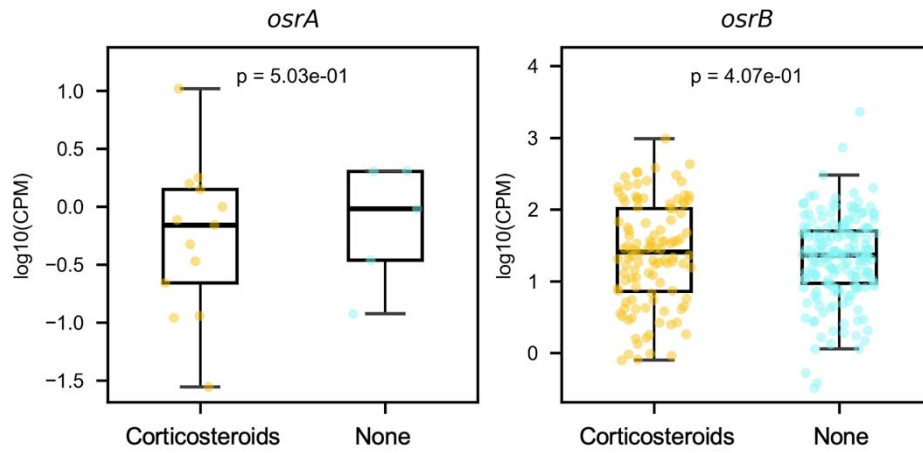
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**Extended Data Figure 7. *osrA* levels in expanded dataset of healthy and Crohn's disease metagenomes.** (A) Reads mapping to *osrA* homologs in expanded dataset of Crohn's disease (CD) patient metagenomes relative to healthy controls. (B) Difference in *osrA* homolog levels in CD relative to healthy metagenomes.

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 818 **Extended Data Figure 8. Association between *osrA* and *osrB* and Crohn's disease in Integrative**  
 819 **Human Microbiome Project metagenomes.** (A) Reads mapping to *osrA* and *osrB* homologs in Crohn's  
 820 disease (CD) metagenomes relative to non-IBD controls. CPM stands for copies per million. (B)  
 821 Difference in *osrB* homolog levels from taxa with the most significant changes in relative abundance  
 822 between CD dysbiotic and non-IBD metagenomes. (C) Difference in *osrA* homolog levels in CD dysbiotic  
 823 relative to non-IBD metagenomes.



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**Extended Data Figure 9. Association between *osrA* and *osrB* and steroid usage in the Lewis et al study.** Reads mapping to *osrA* and *osrB* homologs in Crohn's disease (CD) patient metagenomes grouped based on patient corticosteroid treatment.